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JC759 U.S. PTO 03/09/00

UTILITY PATENT APPLICATION TRANSMITTAL

Attorney Docket No,	96-0296-96544
First Inventor or Application Identifier	James A. Thomson
Title	SERUM FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS
Express Mail Label No,	EJ636878508US

(Only for new nonprovisional applications under 37 CFR 1.53(b))

JC678 U.S. PTO 09/520030 03/09/00

APPLICATION ELEMENTS

ADDRESS TO: Assistant Commissioner for Patents, Box Patent Application

1 [X] Fee transmittal Form (Submit an original and a duplicate for fee processing)

2 [X] Specification [Total Pages 17] *(preferred arrangement set forth below)*

- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed Sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 [ ] Drawing(s) (35 USC 113) [Total Sheets]

4. Oath or Declaration [Total Pages 2]

a. [X] Newly executed (original or copy)

b. [ ] Copy from prior Application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)

[Note Box 5 below]

i. [ ] DELETION OF INVENTOR(S)

inventor(s) named in prior application,

5 [ ] Incorporation By Reference *(useable if Box 4b is checked)* The entire disclosure of the prior application from

under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference herein.

6. [ ] Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*

a. [ ] Computer readable Copy
b. [ ] Paper Copy (identical to computer copy)
c. [ ] Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

8 [ ] Assignment Papers (cover sheet & documents)

9 [ ] 37 CFR 3.73(b) Statement (where there is an assignee) [X] Power of Attorney

10 [ ] English Translation Document *(if applicable)*

11 [X] Information Disclosure Statement (IDS)/PTO-1449 [X] Copies of IDS Citations

12 [ ] Preliminary Amendment

13 [X] Return receipt postcard (MPEP 503) *(Should be specifically itemized)*

14 [X] *Small Entity Statement(s) [ ] Statement filed in prior application Status still proper and desired

15 [ ] Certified copy of priority Document(s)

16 [ ] Other:

* A newstatement is required to pay small entity fees, except where

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application no. _____

Prior application information: Examiner: Group/Art Unit:

18. CORRESPONDENCE ADDRESS

[ ] Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label) or [X] Correspondence address below

NAME	Carl R. Schwartz				
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Name (Print/Type)	Carl R. Schwartz	Registration No. (Attorney/Agent)	29,437
Signature		Date	March 9, 2000

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Attorney's Docket No. ____________________

Applicant or Patentee: James A. Thomson et al.

Serial or Patent No.: ____________________

Filed or Issued: ____________________

For: METHOD FOR SERUM-FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled METHOD FOR SERUM-FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

described in

☒ the specification filed herewith.

☐ application serial no. ____________, filed ____________.

☐ patent no. ____________, issued ____________.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern, or organization

☒ persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: Wisconsin Alumni Research Foundation

ADDRESS: P.O. Box 7365, Madison, WI 53707-7365

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME: ____________________

ADDRESS: ____________________

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

James A. Thomson

Name of inventor

James A Thomson

Signature of inventor

Date 1/7/00

Attorney's Docket No.

Applicant or Patentee: James A. Thomson et al. ______________________

Serial or Patent No.: ______________________

Filed or Issued: ______________________

For: METHOD FOR SERUM-FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Wisconsin Alumni Research Foundation

ADDRESS OF ORGANIZATION: P.O. Box 7365, Madison, WI 53707-7365

TYPE OF ORGANIZATION

☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))

☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA

(NAME OF STATE ______________________)

(CITATION OF STATUTE ______________________)

☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

(NAME OF STATE ______________________)

(CITATION OF STATUTE ______________________)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled METHOD FOR SERUM-FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

by inventor(s) James A. Thomson et al.

described in

☒ the specification filed herewith.

☐ application serial no. __________, filed ____________________.

☐ patent no. __________, issued ____________________.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME: ____________________

ADDRESS: ____________________

____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: ____________________

ADDRESS: ____________________

____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Bryan Z. Renk

TITLE IN ORGANIZATION Director of Patents and Licensing

ADDRESS OF PERSON SIGNING Wisconsin Alumni Research Foundation

P.O. Box 7365, Madison, WI 53707-7365

SIGNATURE Bryan Z. Renk Date 3/3/00

SERUM FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

CROSS REFERENCES TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

The present invention relates to methods for culturing primate embryonic stem cell cultures and culture media useful therewith.

Primate (e.g. monkey and human) pluripotent embryonic stem cells have been derived from preimplantation embryos. See U.S. patent 5,843,780 and J. Thomson et al., 282 Science 1145-1147 (1998). The disclosure of these publications and of all other publications referred to herein are incorporated by reference as if fully set forth herein. Notwithstanding prolonged culture, these cells stably maintain a developmental potential to form advanced derivatives of all three embryonic germ layers.

Primate (particularly human) ES cell lines have widespread utility in connection with human developmental biology, drug discovery, drug testing, and transplantation medicine. For example, current knowledge of the post-implantation human embryo is largely based on a limited number of static histological sections. Because of ethical considerations the underlying mechanisms that control the developmental decisions of the early human embryo remain essentially unexplored.

Although the mouse is the mainstay of experimental mammalian developmental biology, and although many of the fundamental mechanisms that control development are conserved between mice and humans, there are significant differences between early mouse and human development. Primate/human ES cells should therefore provide important new insights into their differentiation and function.

Differentiated derivatives of primate ES cells could be used to identify gene targets for new drugs, used to test toxicity or teratogenicity of new compounds, and used for transplantation to replace cell populations in disease. Potential conditions that might be treated by the transplantation of ES cell-derived cells include Parkinson's disease, cardiac infarcts, juvenile-onset diabetes mellitus, and leukemia. See e.g. J. Rossant et al. 17 Nature Biotechnology 23-4 (1999) and J. Gearhart, 282 Science 1061-2 (1998).

Long term proliferative capacity, developmental potential after prolonged culture, and karyotypic stability are key features with respect to the utility of primate embryonic stem cell cultures. Cultures of such cells (especially on fibroblast feeder layers) have typically been supplemented with animal serum (especially fetal bovine serum) to permit the desired proliferation during such culturing.

For example, in U.S. patents 5,453,357, 5,670,372 and 5,690,296 various culture conditions were described, including some using a type of basic fibroblast growth factor together with animal serum. Unfortunately, serum tends to have variable properties from batch to batch, thus affecting culture characteristics.

In WO 98/30679 there was a discussion of providing a serum-free supplement in replacement for animal serum to support the growth of certain embryonic stem cells in culture. The serum replacement included albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. It was noted that this replacement could be further supplemented with leukemia inhibitory factor, steel factor, or ciliary

neurotrophic factor. Unfortunately, in the context of primate embryonic stem cell cultures (especially those grown on fibroblast feeder layers), these culture media did not prove satisfactory.

In the context of nutrient serum culture media (e.g. fetal bovine serum), WO 99/20741 discusses the benefit of use of various growth factors such as bFGF in culturing primate stem cells. However, culture media without nutrient serum is not described.

In U.S. patent 5,405,772 growth medium for hematopoietic cells and bone marrow stromal cells are described. There is a suggestion to use fibroblast growth factor in a serum-deprived media for this purpose. However, conditions for growth primate of embryonic stem cells are not described.

It can therefore be seen that a need still exists for techniques to stably culture primate embryonic stem cells without the requirement for use of animal serum.

BRIEF SUMMARY OF THE INVENTION

In one aspect the invention provides a method of culturing primate embryonic stem cells. One cultures the stem cells in a culture essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer. In a preferred form the culture also has a fibroblast feeder layer.

Fibroblast growth factors are essential molecules for mammalian development. There are currently nine known fibroblast growth factor ligands and four signaling fibroblast growth factor receptors therefor (and their spliced variants). See generally D. Ornitz et al., 25 J. Biol. Chem. 15292-7 (1996); U.S. patent 5,453,357. Slight variations in these factors are expected to exist between species, and thus the term fibroblast growth

factor is not species limited. However, I prefer to use human fibroblast growth factors, more preferably human basic fibroblast growth factor produced from a recombinant gene. This compound is readily available in quantity from Gibco BRL-Life Technologies and others.

It should be noted that for purposes of this patent the culture may still be essentially free of the specified serum even though a discrete component (e.g. bovine serum albumin) has been isolated from serum and then is exogenously supplied. The point is that when serum itself is added the variability concerns arise. However, when one or more well defined purified component(s) of such serum is added, they do not.

Preferably the primate embryonic stem cells that are cultured using this method are human embryonic stem cells that are true ES cell lines in that they: (i) are capable of indefinite proliferation <u>in vitro</u> in an undifferentiated state; (ii) are capable of differentiation to derivatives of all three embryonic germ layers (endoderm, mesoderm, and ectoderm) even after prolonged culture; and (iii) maintain a normal karyotype throughout prolonged culture. They are therefore referred to as being pluripotent.

The culturing permits the embryonic stem cells to stably proliferate in culture for over one month (preferably over six months; even more preferably over twelve months) while maintaining the potential of the stem cells to differentiate into derivatives of endoderm, mesoderm, and ectoderm tissues, and while maintaining the karyotype of the stem cells.

In another aspect the invention provides another method of culturing primate embryonic stem cells. One cultures the stem cells in a culture essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of a growth

factor capable of activating a fibroblast growth factor signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer. While the growth factor is preferably a fibroblast growth factor, it might also be other materials such as certain synthetic small peptides (e.g. produced by recombinant DNA variants or mutants) designed to activate fibroblast growth factor receptors. See generally T. Yamaguchi et al., 152 Dev. Biol. 75-88 (1992)(signaling receptors).

In yet another aspect the invention provides a culture system for culturing primate embryonic stem cells. It has a fibroblast feeder layer and human basic fibroblast growth factor supplied by other than just the fibroblast feeder layer. The culture system is essentially free of animal serum.

Yet another aspect of the invention provides cell lines (preferably cloned cell lines) derived using the above method. "Derived" is used in its broadest sense to cover directly or indirectly derived lines.

Variability in results due to differences in batches of animal serum is thereby avoided. Further, it has been discovered that avoiding use of animal serum while using fibroblast growth factor can increase the efficiency of cloning.

It is therefore an advantage of the present invention to provide culture conditions for primate embryonic stem cell lines where the conditions are less variable and permit more efficient cloning. Other advantages of the present invention will become apparent after study of the specification and claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following experiments I used the methods and culture systems of the invention to culture human ES cell lines. Two clonally derived human ES cell lines proliferated for over eight months after clonal

derivation and maintained the ability to differentiate to advanced derivatives of all three embryonic germ layers.

Techniques for the initial derivation, culture, and characterization of the human ES cell line H9 were described in J. Thomson et al., 282 Science 1145-1147 (1998). In my experiments herein human ES cells were then plated on irradiated (35 gray gamma irradiation) mouse embryonic fibroblast. Culture medium for the present work consisted of 80% "KnockOut" Dulbeco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD), 1 mM L-Glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids stock (Gibco BRL, Rockville, MD), supplemented with either 20% fetal bovine serum (HyClone, Logan, UT) or 20% KnockOut SR, a serum-free replacement originally optimized for mouse ES cells (Gibco BRL, Rockville, MD). The components of KnockOut SR are those described for serum replacements in WO 98/30679.

In alternative experiments medium was supplemented with either serum or the aforesaid serum replacer KnockOut SR, and either with or without human recombinant basic fibroblast growth factor (bFGF, 4 ng/ml). The preferred concentration range of bFGF in the culture is between .1 ng/ml to 500 ng/ml.

To determine cloning efficiency under varying culture conditions, H-9 cultures were dissociated to single cells for 7 minutes with 0.05% trypsin/0.25% EDTA, washed by centrifugation, and plated on mitotically inactivated mouse embryonic fibroblasts (10^5 ES cells per well of a 6-well plate). To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96 well plate containing mouse

embryonic fibroblasts feeders with medium containing 20% serum replacer and 4 ng/ml bFGF.

Clones were expanded by routine passage every 5-7 days with 1 mg/ml collagenase type IV (Gibco BRL, Rockville, MD). Six months after derivation, H9 cells exhibited a normal XX karyotype by standard G-banding techniques (20 chromosomal spreads analyzed). However, seven months after derivation, in a single karyotype preparation, 16/20 chromosomal spreads exhibited a normal XX karyotype, but 4/20 spreads demonstrated random abnormalities, including one with a translocation to chromosome 13 short arm, one with an inverted chromosome 20, one with a translocation to the number 4 short arm, and one with multiple fragmentation. Subsequently, at 8, 10, and 12.75 months after derivation, H9 cells exhibited normal karyotypes in all 20 chromosomal spreads examined.

We observed that the cloning efficiency of human ES cells in previously described culture conditions that included animal serum was poor (regardless of the presence or absence of bFGF). We also observed that in the absence of animal serum the cloning efficiency increased, and increased even more with bFGF.

The data expressed below is the total number of colonies resulting from 10^5 individualized ES cells plated, +/- standard error of the mean (percent colony cloning efficiency). With 20% fetal serum and no bFGF there was a result of 240 +/- 28. With 20% serum and bFGF the result was about the same, 260 +/- 12. In the absence of the serum (presence of 20% serum replacer) the result with no bFGF was 633 +/- 43 and the result with bFGF was 826 +/- 61. Thus, serum adversely affected cloning efficiency, and the presence of the bFGF in the absence of serum had an added synergistic benefit insofar as cloning efficiency.

The long term culture of human ES cells in the presence of serum does not require the addition of exogenously supplied bFGF, and (as noted above) the addition of bFGF to serum-containing medium does not significantly increase human ES cell cloning efficiency. However, in serum-free medium, bFGF increased the initial cloning efficiency of human ES cells.

Further, I have discovered that supplying exogenous bFGF is very important for continued undifferentiated proliferation of primate embryonic stem cells in the absence of animal serum. In serum-free medium lacking exogenous bFGF, human ES cells uniformly differentiated by two weeks of culture. Addition of other factors such as LIF (in the absence of bFGF) did not prevent the differentiation.

The results perceived are particularly applicable to clonal lines. In this regard, clones for expansion were selected by placing cells individually into wells of a 96 well plate under direct microscopic observation. Of 192 H-9 cells plated into wells of 96 well plates, two clones were successfully expanded (H-9.1 and H-9.2). Both of these clones were subsequently cultured continuously in media supplemented with serum replacer and bFGF.

H9.1 and H9.2 cells both maintained a normal XX karyotype even after more than 8 months of continuous culture after cloning. The H-9.1 and H-9.2 clones maintained the potential to form derivatives of all three embryonic germ layers even after long term culture in serum-free medium. After 6 months of culture, H9.1 and H9.2 clones were confirmed to have normal karyotypes and were then injected into SCID-beige mice.

Both H9.1 and H9.2 cells formed teratomas that contained derivatives of all three embryonic germ layers including gut epithelium (endoderm) embryonic kidney, striated muscle, smooth muscle, bone, cartilage

(mesoderm), and neural tissue (ectoderm). The range of differentiation observed within the teratomas of the high passage H9.1 and H9.2 cells was comparable to that observed in teratomas formed by low passage parental H9 cells.

It should be appreciated from the description above that while animal serum is supportive of growth it is a complex mixture that can contain compounds both beneficial and detrimental to human ES cell culture. Moreover, different serum batches vary widely in their ability to support vigorous undifferentiated proliferation of human ES cells. Replacing serum with a clearly defined component reduces the variability of results associated with this serum batch variation, and should allow more carefully defined differentiation studies.

Further, the lower cloning efficiency in medium containing serum suggests the presence of compounds in conventionally used serum that are detrimental to stem cell survival, particularly when the cells are dispersed to single cells. Avoiding the use of these compounds is therefore highly desired.

The present invention has been described above with respect to its preferred embodiments. Other forms of this concept are also intended to be within the scope of the claims. For example, while recombinantly produced human basic fibroblast growth factor was used in the above experiments, naturally isolated fibroblast growth factor should also be suitable. Further, these techniques should also prove suitable for use on monkey and other primate cell cultures.

Thus, the claims should be looked to in order to judge the full scope of the invention.

Industrial Applicability

The present invention provides methods for culturing primate embryonic stem cells, and culture media for use therewith.

CLAIMS

I claim:

1. A method of culturing primate embryonic stem cells, comprising:

culturing the stem cells in a culture essentially free of mammalian fetal serum and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer.

2. The method of claim 1, wherein the culture is essentially free of any animal serum.

3. The method of claim 2, wherein the culture also comprises a fibroblast feeder layer.

4 The method of claim 2, wherein the fibroblast growth factor is basic fibroblast growth factor.

5. The method of claim 4, wherein the fibroblast growth factor is human basic fibroblast growth factor which has been produced from a recombinant gene.

6. The method of claim 2, wherein the primate embryonic stem cells are human embryonic stem cells.

7. The method of claim 2, wherein said culturing step includes the embryonic stem cells proliferating in culture for over one month while maintaining the potential of the stem cells to differentiate into derivatives of endoderm, mesoderm, and ectoderm tissues, and while maintaining the karyotype of the stem cells.

8. The method of claim 2, wherein the human basic fibroblast growth factor is present in the culture in a concentration of at least .1 ng/ml for at least a portion of the method.

9. A method of culturing primate embryonic stem cells, comprising:

culturing the stem cells in a culture essentially free of mammalian fetal serum and in the presence of a growth factor capable of activating a fibroblast growth factor signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer.

10. The method of claim 9, wherein the culture is essentially free of any animal serum.

11. The method of claim 10, wherein the culture also comprises a fibroblast feeder layer.

12. The method of claim 10, wherein the primate embryonic stem cells are human embryonic stem cells.

13. The method of claim 10, wherein said culturing step includes the embryonic stem cells proliferating in culture for over one month while maintaining the potential of the stem cells to differentiate into derivatives of endoderm, mesoderm, and ectoderm tissues, and while maintaining the karyotype of the stem cells.

14. A culture system for culturing primate embryonic stem cells, comprising:

a fibroblast feeder layer; and

fibroblast growth factor supplied by other than just the fibroblast layer;

wherein the culture system is essentially free of animal serum.

15. A cell line derived using the method of claim 1.

16. A cell line derived using the method of claim 9.

ABSTRACT

Disclosed herein are methods for culturing primate embryonic stem cells. These cells are cultured on a prolonged and stable basis in the presence of exogenously supplied fibroblast growth factor and in the absence of animal serum. Preferably there is also a fibroblast feeder layer. Also disclosed is a culture media containing fibroblast feeder layer and the fibroblast growth factor.

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PTO/SB/01 (6-95)
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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

☒ Declaration Submitted with Initial Filing OR ☐ Declaration Submitted after Initial Filing

Attorney Docket Number	960296.96544
First Named Inventor	James A. Thomson
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SERUM FREE CULTIVATION OF PRIMATE EMBRYONIC STEM CELLS

(Title of the Invention)

the specification of which

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
N/A			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
		☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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Please type a plus sign (+) inside this box [+]

DECLARATION

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

[ ] Firm Name _____ Customer Number or label _____

OR

[X] List attorney(s) and/or agent(s) name and registration number below

Name	Registration Number	Name	Registration Number
Carl R. Schwartz	29,437	Jean C. Baker David G. Ryser	35,433 36,407

[ ] Additional attorney(s) and/or agents named on a supplemental priority sheet attached hereto

Please direct all correspondence to [ ] Customer Number or label _____ OR [X] Fill in correspondence address below

Name	Carl R. Schwartz				
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor: [ ] A petition has been filed for this unsigned inventor

Given	James	Middle	A	Family	Thomson	Suffix	
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[ ] Additional inventors are being named on supplemental sheet(s) attached hereto